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ISSR (Inter Simple Sequence Repeats) as molecular markers to study genetic diversity in tarantulas (Araneae, Mygalomorphae)

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Abstract. Although all species of the *Brachypelma* genus are protected under CITES, few studies have been performed on the genetic structure of the populations of these endangered tarantulas. Here we propose, for the first time in spiders, to use ISSR (Inter Simple Sequence Repeat) technique to study the genetic variability of Mexican populations of *Brachypelma vagans* (Ausserer 1875). We used a nonlethal technique to collect samples from six populations in the Yucatan peninsula and we tested seven ISSR primers. Four of these primers gave fragments (bands) that were sufficiently clear and reproducible to construct a binary matrix and determine genetic variability parameters. We revealed a very high percentage of polymorphism ($P = 98.7\%$) the highest yet reported for tarantula spiders. Our results show that the ISSR-PCR method is promising for intraspecific variation of tarantula spiders.

Keywords: ISSR, Theraphosinae, *Brachypelma*, genetic population, Mexican redrump tarantula

Members of the genus *Brachypelma* are charismatic spiders, being colorful, large, and docile (Locht et al. 1999). The pet trade, habitat destruction, high mortality rates as juveniles, and late sexual maturity result in all *Brachypelma* species being listed in Appendix II of CITES. In recent years, efforts have been made to increase knowledge of their ecology (Yáñez & Floater 2000; Machkour-M'Rabet et al. 2005, 2007) and behavior (Locht et al. 1999; Reichling 2000). However, studies to better understand the genetic structure of tarantula populations are essential to assess the conservation status of the genus. Recently, the development of molecular techniques has helped inform conservation strategies.

Here, we focused our effort on *Brachypelma vagans* (Ausserer 1875), which is distributed from Southern Mexico south to Costa Rica (Locht et al. 1999), but has also been recorded outside its natural range in Florida as a result of the release of pet trade animals (Edwards & Hibbard 1999). As with the study of most tarantulas, the biology and ecology of *B. vagans* is poorly known (Carter 1997; Yáñez et al. 1999; Machkour-M'Rabet et al. 2005, 2007) and little information exists on the genetic structure of its populations (Longhorn et al. 2007).

Mitochondrial DNA and allozyme electrophoresis have been used previously to evaluate population genetics in Mygalomorphae (Ramirez & Froehlig 1997; Bond et al. 2001; Pedersen & Loeschcke 2001; Ramirez & Chi 2004; Bond et al. 2006; Arnedo & Ferrández 2007). For the *Brachypelma* genus (Theraphosidae) only one study has been carried out recently (Longhorn et al. 2007), which focused on the genetic structure of two Belizean populations of *B. vagans* using two portions of mitochondrial DNA (partial 16SRNA + tRNA-Leu + partial ND1 and CO1) and one nuclear non-coding gene (ITS-2). This study showed that nuclear markers are relatively invariant across *B. vagans* populations while mitochondrial markers possess sufficient resolution to estimate the genetic structure of this species. However, it has been suggested that alternative sources of nuclear genes could be used to enhance the characterization of population structure in tarantula spiders. In this context, and because no microsatellite primers are available for *Brachypelma*, we here explore the usefulness of a relatively novel technique in animals, Inter Simple Sequence Repeats (ISSR), to discriminate among populations.

Dominant ISSR markers are widely used in the conservation of rare plants (Kothera et al. 2007) and are being increasingly used in animals (Wink et al. 2002; Hoffman et al. 2006; Guicking et al. 2006; Joger et al. 2007), particularly invertebrates (Abbot 2001; Luque et al. 2002; Chatterjee & Mohandas 2003; Hundsdoerfer & Wink 2006; Roux et al. 2007). However, until now, this technique has not been applied to spiders.

The PCR-ISSR method was used here to screen a large part of the genome without prior knowledge of the sequences. This provides highly reproducible results and generates abundant polymorphisms. The great advantage of ISSR is that the primers work universally for many animal and plant species. Consequently, it is not necessary to define PCR primers for each species, unlike microsatellites. Furthermore, ISSR demands fewer experimental steps and is therefore easy to carry out with a low cost-benefit ratio compared with RFLP (Restriction Fragment Length Polymorphism) and results in a higher reliability and repeatability than RAPD (Random Amplification of Polymorphic DNA; Nagaraju et al. 2001; Luque et al. 2002). Absence of a band is interpreted as the loss of a locus through either the deletion of the SSR (Simple Sequence Repeat) site or a chromosomal rearrangement (Wolfe & Liston 1998). ISSR are thus considered and treated as dominant markers (Casu et al. 2005).

The method uses polymerase chain reaction (PCR) with repeat-anchored or non-anchored primers to amplify DNA sequences between two inverted SSR (Zietkiewicz et al. 1994).

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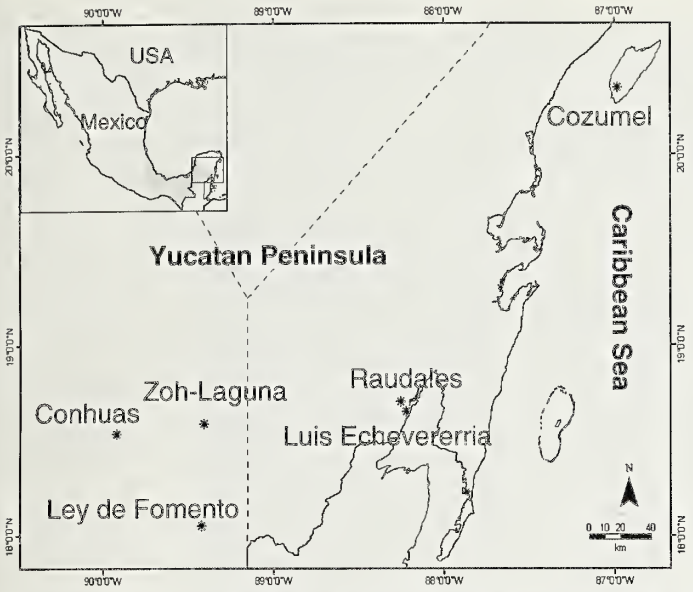

Figure 1.—Location of the six samples used in the study in the Yucatan Peninsula (represented by stars). The geographic distribution of the Mexican Redrump Tarantula, *Brachypelma vagans*, in Mexico is represented on the smaller map (upper left).

Therefore, the only DNA stretches amplified are positioned between two identical but inverted microsatellites (SSR). A single primer can amplify up to 80 loci simultaneously. This method provides genomic information available for a broad spectrum of applications: population genetics, hybridizations, and gene mapping (Wink 2006). The ISSR method represents one of the most promising tools in population genetics studies and deserves increased attention (Behura 2006).

The aim of this study was to technically adapt ISSR-PCR for tarantulas and to provide a preliminary assessment of whether this method is advantageous to explore the genetic structure of tarantula populations. The only previous study using dominant markers (RAPD; Hettle et al. 1997) to study genetic populations of tarantula spiders (*Brachypelma albopilosum* Valerio 1980) showed that none of the six primers used were reliable to differentiate inter- and intra-family relationships. Here we report the technical aspects of a recent molecular tool to study populations of endangered tarantulas.

METHODS

Brachypelma vagans samples were collected during March and April 2007 around six traditional villages of the Yucatan Peninsula (Mexico): Ley de Fomento: 18°03′N, 89°25′W; Conhuas: 18°32′N, 89°55′W; Zoh-Laguna: 18°35′N, 89°24′W; Luis Echevererria: 18°39N, 88°13′W; Raudales: 18°42′N, 88°15′W; and Cozumel: 20°21′N, 86°59′W (Fig. 1). We collected the samples using a nonlethal technique that consists of inducing limb autotomy (Longhorn 2002). In response to pressure, the limb will detach and the muscles will contract to prevent hemolymph loss; spiders in which a limb is removed will regenerate the limb during subsequent molts. We chose to remove the medial limb (III) because the anterior legs (I and II) are used in sensory behaviors and the posterior legs (IV) are used defensively in brushing urticating hairs (Smith 1994). Samples were preserved in 95% ethanol at room temperature, and sent for DNA analysis under CITES export permit (MX34176) to ECOLAB (Laboratoire d'Ecologie Fonctionnelle) at University Paul Sabatier (Toulouse, France).

A small part of the limb was cut off and incubated for 12 h at 50°C in 350 µl of buffer B (10 mM Tris, pH 7.5, 25 mM EDTA, and 75 mM NaCl) with 500 µg of proteinase K and 20 µl of SDS (20%). Proteins and residues were precipitated with 200 µl of saturated NaCl solution and centrifuged at 14,000 rpm for 30 min. DNA from the supernatant was saved and precipitated with 400 µl of cold isopropanol, mixed and centrifuged at 14,000 rpm for 40 min at 2° C. The isopropanol was eliminated and the precipitate was washed with 500 µl of 70% ethanol and centrifuged at 14,000 rpm for 10 min at 2° C. The precipitate was dried and redissolved in 100 µl of TE buffer (pH = 7) and preserved at −28° C until utilization. The concentration of the DNA obtained was determined by spectrophotometry (NanoDrop ND-1000) and the quality was checked using electrophoresis in agarose/TBE (1.2%) gel.

Inter Simple Sequence Repeat (ISSR) analysis was performed using seven primers (Table 1). PCR amplifications were performed in a 25 µl reaction volume containing ~20 ng of template DNA, 50 µM of primer (Invitrogen), 0.2 mM of each dNTP from dNTP Mix (Promega), 2.5 µl of 5× Green Buffer (Promega), 3 µl of $MgCl_2$ (1.5 mM, Promega), and 2.5 U of *Taq* polymerase (Promega). All amplifications were done in a T3 Thermocycler (Biometra). The cycling conditions were as follows: initial denaturation step at 94° C for 4 min, 39 cycles of denaturation at 94° C for 45 s, primer annealing at 56° C for 45 s, and extension at 72° C for 2 min, followed by a final extension at 72° C for 10 min.

Electrophoresis was performed with 7 µl of amplified products on a 2% agarose gel using 1× Tris acetate EDTA buffer at 140 V for ~2 h. The bands were detected with ethidium bromide under UV light and digitized (Bio-Vision 3000, Vilbert-Lourmat) (Figure 2).

In our first experiments, conditions for ISSR with different primers were not optimal. One of the most important factors is

Table 1.—SSR primers screened for ISSR-PCR in the tarantula *Brachypelma vagans*. B = T, C or G; D = A, T or G; R = A or G; Y = C or T; W = A or T.

Code	Sequence (5′→3′)	Abbreviation	Amplification pattern	Total bands
CA	CACACACACACACA	$(CA)_7$	Poor amplification	-
CA+	CACACACACACACARY	$(CA)_7RY$	Smeared	-
+CA	RYCACACACACACACA	$RY(CA)_7$	Smeared with band	-
ACA+	ACAACAACAACAACABDB	$(ACA)_5BDB$	Good	16
+ACA	BDBACAACAACAACAACA	$BDB(ACA)_5$	Good	25
GACA+	GACAGACAGACAGACAWB	$(GACA)_4WB$	Good	15
+GACA	WBGACAGACAGACAGACA	$WB(GACA)_4$	Good	20

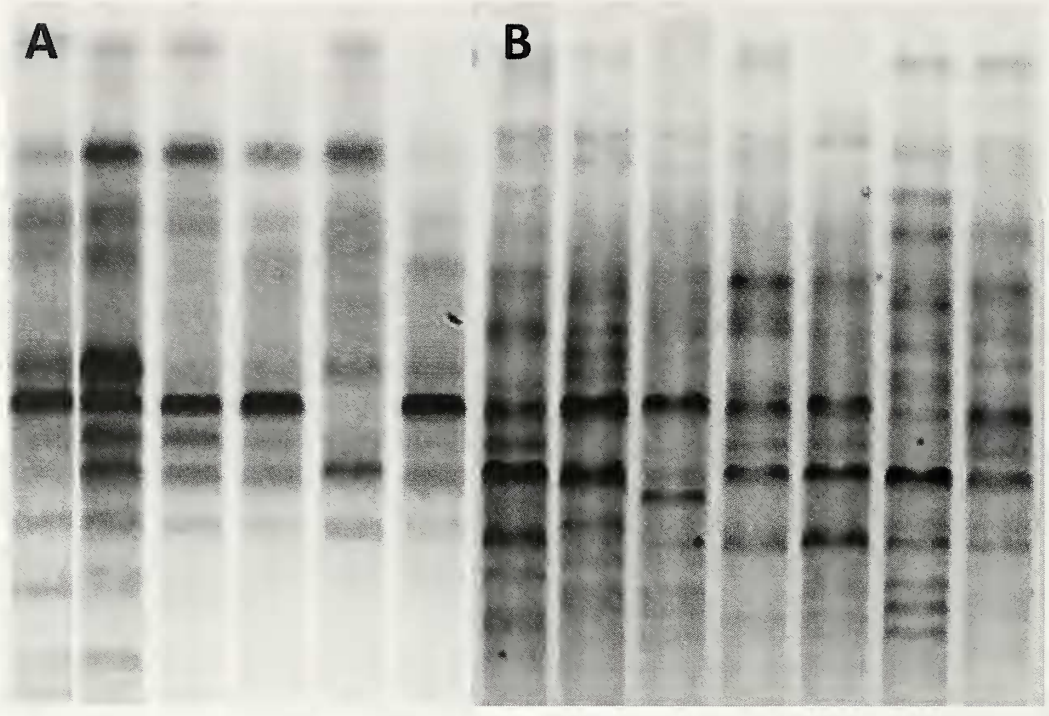

Figure 2.—Example of polymorphic ISSR banding patterns with one marker (+ACA) for two different populations: Cozumel (A) and Luis Echevererria (B).

the difference in the amount of DNA loaded that can weaken the quality of the electrophoretic resolution. In order to obtain comparable and reliable results, we used the same DNA concentration for all samples. We found a minimum optimal amount of 20 ng per sample. Another important parameter is the primer annealing temperature. We experimented with temperatures ranging from 46° C to 66° C with a step of 1° C for all primers and we chose an optimal temperature (56° C) identical for all primers to facilitate the PCR procedure. Also, we checked various parameters to optimize our results. The standard number of reamplifications (39 cycles) was used and gave repeatable and reliable results for all primers. The concentration of $MgCl_2$ was tested from 2.6 µl to 3.4 µl in steps of 0.2 µl and we found good results (quality of the electrophoretic resolution) for values above 3 µl. The concentration of buffer was checked from 2.1 µl to 2.9 µl in steps of 0.2 µl and these modifications had no influence on the results, then we chose a medium value of 2.5 µl. Finally, the method used for storage of the spiders limbs [i.e. preservation in ethanol (95%) and dry-conservation at room temperature (3 years old)], was checked. Only preservation in ethanol resulted in amplification.

The gel separation of ISSR fragments (bands) was used for each individual and each primer to score the presence (1) or absence (0) of bands. This information generated the binary matrix used for analysis. Only bands that could be scored consistently among populations were used, and we assumed that each marker band represented a distinct locus.

The binary matrix was used under Hardy-Weinberg equilibrium to determine the genetic diversity: percentage of polymorphism (*P*), Nei's gene diversity (*h*) using corrected allele frequency (Lynch & Milligan 1994) and the Shannon Index (*H*) (Lewontin 1972), at the species level and for each population. All analyses were carried out using POPGEN Version 1.32 (Yeh et al. 1997). In order to describe the genetic structure and variability among and between populations, non-parametric Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) was performed with GENALEX V6 (9999 permutations; Peakall & Smouse 2006).

RESULTS

Of the seven primers initially tested for the six populations, only four produced clear reproducible fragments (Table 2). Interestingly, the most classic and polymorphic primer (CA_n)

Table 2.—Genetic diversity of *Brachypelma vagans* in the Yucatan Peninsula based on ISSR markers. *n*: number of individuals kept for analysis; N^1: number of bands scored; N^2: number of polymorphic bands; N^3: number of signature bands; *P*: percentage of polymorphism; *h*: Nei's gene diversity; *H*: Shannon Index; SD: standard deviation.

Population name	*n*	N^1	N^2	N^3	*P* (%)	*h* (± SD)	*H* (± SD)
Raudales	22	64	61	1	80.26	0.273 (0.181)	0.411 (0.252)
Zoh-Laguna	24	65	58	0	76.32	0.272 (0.191)	0.405 (0.268)
Ley de Fomento	26	69	64	2	84.21	0.296 (0.178)	0.442 (0.243)
Conhuas	23	65	59	3	77.63	0.271 (0.196)	0.403 (0.270)
Luis Echevererria	26	64	56	1	73.68	0.284 (0.197)	0.418 (0.277)
Cozumel	27	56	43	0	56.58	0.193 (0.203)	0.288 (0.291)

for butterflies (Nagaraju et al. 2001; Luque et al. 2002; Hundsdoerfer & Wink 2006; Roux et al. 2007) failed in the tarantula. From these four primers, a total of 76 scorable ISSR fragments were selected in the 180 individuals screened from all populations (30 individuals for each population). In Table 2, the number of bands and the number of polymorphic bands for each population is given. In addition, we give the number of bands found only within each of the populations, which we call "diagnostic bands" (Table 2; Luque et al. 2002). The very low number of these bands indicates that all populations belong to the same species.

Of the 30 individuals of each population, we only kept individuals presenting a banding pattern for the four primers and for which the interpretation of the banding pattern was unequivocal. For this reason, the number of individuals used for analysis is lower than the number screened (Table 2).

The percentage of polymorphic loci (P) varied between populations (Table 2), ranging from 57% in the Cozumel island population to 84% in the Ley de Fomento population. A mean P of 98.7% was observed across the 6 populations. Nei's gene diversity (h) was low in the Cozumel population with 0.193 (SD = 0.203), while it was higher but relatively constant for continental populations (Table 2). The mean for all populations was 0.324 (SD = 0.164). For the Shannon Index (H), we observed a similar pattern (Table 2): Cozumel island diversity was lower, with a mean across all populations of 0.485 (SD = 0.213).

AMOVA analysis revealed that 79% ($df = 142$, $P < 0.001$) of the variability occurred among individuals within populations and that a strong genetic difference among populations was observed (21%, $df = 5$).

DISCUSSION

Our study revealed a high level of polymorphism for tarantulas in comparison with other studies using allozymes [$P < 7.7\%$ for *Aptostichus simus* Chamberlin 1917 (Cyrtaucheniidae), Ramirez & Froehlig 1997; $P < 33\%$ for *Atypus affinis* Eichwald 1830 (Atypidae), Pedersen & Loeschcke 2001; $P < 30\%$ for *Antrodiaetus riversi* (O. Pickard-Cambridge 1883) (Antrodiaetidae), Ramirez & Chi 2004 (reported therein as *Atypoides riversi*)]. However, the allozyme technique is known to detect a low level of polymorphism with regard to other molecular techniques, and underestimate gene variation (Lowe et al. 2004). Genetic diversity values obtained in our study are congruent with a species having open populations and ample distribution with high gene flow probabilities (Roux et al. 2007; Bouzid et al. 2008).

Consequently, the choice of appropriate molecular markers is very important to study genetic variation at the intraspecific level. In the present study, all mainland populations presented high and similar levels of polymorphism and gene diversity coefficients, whereas the island population of Cozumel presented the lowest values. Generally founded from a small number of individuals (founder effect), island populations usually present less genetic diversity than mainland populations and are often inbred (limited gene flow) (Frankham et al. 2005). However, the values of the Cozumel population did not indicate a threatened population and suggest recent colonization of the island, or an ancient colonization with the occasional introduction, most likely by man, of new individuals from the mainland that can decrease the genetic drift effect.

This study clearly showed the potential of ISSR markers to evaluate genetic diversity in tarantula spiders, and proved an attractive alternative to other molecular markers.

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